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Phytochemical Constituents and Bioscreening Activities of Alexandria Mediterranean Sea Green and Red Algae

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ABSTRACT

Seaweeds, besides having many nutrients, also manifest potentially beneficial properties for the treatment of various diseases. This study was carried out to investigate the bioactivities of green algae (*Ulva lactuca*) and red algae (*Jania rupin* and *Pterocladia capillacea*) that were collected from Abu Kir beach, Alexandria coast. The bioactivity of these algaes' methanolic extracts were evaluated, including cell oxidants status, α-glucosidase and acetylcholinesterase (AChE) activities. In addition, the coagulatory effect was assessed by measuring prothrombin time (PT) and activated partial thromboplastin time (APTT). Our results showed that the green algae, and the two red algae extracts contained flavonoids (8.4, 17.4 and 16.75%), and phenolics (total phenolic content = 1.028, 1.23 and 1.11%, respectively). All the algal extracts showed a significant antioxidant capacity, where they decreased the level of TBARS in liver homogenate or in human seminal plasma and spermatozoa. Furthermore, *J. rupin* extract only inhibited hepatic α-glucosidase activity, while the other two algal extracts acted as enzyme activators. Moreover, these algal extracts showed a potent inhibitory effect toward AChE activity. Finally, they all showed anticoagulation properties by increasing PT and APTT. In conclusion, all three examined algae extracts could be used for the treatment of some cardiovascular diseases. The methanolic extract of green algae is recommended for the treatment of idiopathic male infertility and Alzheimer's disease compared to the methanolic extracts of red algae. In addition, the methanolic extracts of red algae have a potent hepatoprotective action compared to the green algae.

Keywords: AChE, idiopathic male infertility, *Jania rupin*, *Pterocladia capillacea*, TBARS, *Ulva lactuca*
Abbreviations: AChE, acetylcholinesterase; **APTT**, activated partial thromboplastin time; **AD**, Alzheimer's disease; **ACTI**, acetylthiocholine iodide; **DTNB**, 5,5'-dithiobis 2-nitrobenzoic acid; **PT**, prothrombin; **TBA**, thiobarbituric acid; **TCA**, trichloro acetic acid

INTRODUCTION

This study aimed to investigate the bioscreened activities of the methanolic extracts of green algae (*Ulva lactuca*) and two species of red algae (*Jania rupin* and *Pterocladia capillacea*) that were collected from Abu Kir Beach, Alexandria coast.

Plants produce an enormous variety of natural products with highly diverse structures. These products are commonly termed 'secondary metabolites' in contrast to the "primary metabolites" which are essential for plant growth and development (Sirikantaramas *et al*. 2008). Secondary metabolites were formerly regarded as "waste products" without physiological function for the plant. With the emergence of the field of chemical ecology about 30 years ago, it became an evident however, that these natural products fulfill important functions in the interactions between plants and their biotic and abiotic environment. Natural products also have a strong impact on the human culture, and have been used throughout human history as condiments, pigments, and pharmaceuticals (Dearing 2005).

Terrestrial plants were thought to be the first source for the isolation of natural products, but seaweeds can also provide excellent sources of materials that could provide the basis of a new therapeutic agent (Bhakuni *et al.* 1994). Algae are found in abundance in the sea; about 30,000 species of algae are found where there is light and moisture. The majority of red algae and almost all brown algae proliferate in salt water. Many macroscopic green algae like *Codium*, *Caulerpa*, *Ulva* and *Enteromorpha* grow in shallow waters (Erickson 2002).

The "green algae" is the most diverse group of algae, with more than 7000 species growing in a variety of habitats. Green algae contain two forms of chlorophyll to capture light energy to fuel the manufacture of sugars. These algae differentiate in 3 forms: unicellular, colonial or multicellular (Bhakuni *et al*. 1994).

Red algae contain phycoerythrin pigment, which reflects red light and absorbs blue. Because blue light penetrates water to a greater depth than light of longer wavelengths, these pigments allow red algae to photosynthesize and live at relatively greater depths than other algae (Kotta *et al*. 2008).

Marine organisms exhibit a wide range of biological activities: antifertility (Dhar *et al*. 1992), antiviral (Gustafson *et al*. 2004), antifungal and antimicrobial activities of marine organisms have been reported (Savoian *et al.* 2004). Also algal lectin from *Galaxaura marginata* exhibits antibacterial activity (Liao *et al*. 2003). In addition, palmitic acid, which has antitumor activity, has been isolated from *Colpomenia sinuosa* (Heiba *et al.* 1997). Various anticoagulant polysaccharides compounds have been isolated and characterized from marine green algae (Athukorala *et al*. 2006). The carotenoids from *Dunaliella salina*, a green microalga, are capable of maintaining the activity of hepatic enzymes, which are involved in combating reactive oxygen species (ROS) (Murthy *et al.* 2004).

MATERIALS AND METHODS

Material

Acetylthiocholine iodide (ACTI), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), trichloro acetic acid (TCA), berberine chloride, butylated hydroxytoluen (BHT), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), 5,5′-dithiobis 2-nitro-

Received: 1 February, 2010. Accepted: 5 September, 2010.

Original Research Paper

benzoic acid (DTNB) and *p*-nitrophenyl glucoside were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Kits for PT, APTT were bought from Futural System (Rome, Italy). HAM's nutrient reagent Mixture F-10 was bought from Lone Co., UK. All other chemicals and reagents were of the highest quality available commercially.

Red and green algae were collected from the Abu Kir coast and identify by Prof. Dr. Samy Shaalan, Microbiology and Botany Department, Faculty of Science, Alexandria University. The green alga was *Ulva lactuca* while the red algae were *Jania rupin* and *Pterocladia capillacea*.

Methods

The algae washed in tap water were dried in air, separately ground then soaked in methanol overnight. The methanolic extract was collected by vacuum filtration then condensed by a rotary evaporator (Büchi, Switzerland) and lyophilized to obtain the powder form. Each powder was dissolved in 100 mg/100 mL acetic acid, 5% of which was used for further investigations (tested extract).

The presence of flavonoids was assessed according to Kim *et al*. (2003) while determination of total phenolic compounds was carried out according to Singleton *et al.* (1999).

The determination of acetylcholinesterase [AChE; EC 3.1.1.7] activity was determined by the method of Ellman (1961) with modifications; 130 µL of phosphate buffer (0.1 M, pH = 7.4) were added to 20 µL of each tested extract and 20 µL of liver homogenate (10% w/v in phosphate buffer 0.1 M, pH 7.4) and incubated at 37°C for 45 min. Then the Ellman reaction was carried out.

The determination of α-glucosidase [EC 3.2.1.20] activity was carried out as described by Li (2005) with modification; 2.5 mL phosphate buffer (0.1 M, pH = 7.4) was added to 100 µL of each tested extract and 100 µL of liver homogenate (as prepared previously) then incubated at 30°C for 5 min. finally, the enzymatic reaction was carried out by the Han and Srinivasan method (1969).

Determination of prothrombin time (PT) and activated partial thromboplastin time (APTT) (Errchetti *et al*. 1984): 100 µL of each tested extract was added to 100 µL of human serum (blood withdrawal was provided from a healthy subject, with patient consent, under the supervision of Dr. Doaa Ahmed Ghareeb, clinical biochemist, according to Alexandria University Ethics Regulation Book). The mixture was incubated for 5 min at 37°C. Then the method was carried out as described by Errchetti *et al*. (1984).

Determination of liver homogenate thiobarbituric acid-reactive substances (TBARS): 2 mL of each tested extract was added to 2 mL of liver homogenate, and then the mixture was incubated for 45 min at 37°C. 100.25 µL of $FeSO_4 \cdot 7H_2O$ (0.02 M) and 10 µL of H_2O_2 (0.4 M) were added to each sample and incubated for 30 min at 37°C. Finally 80.6 µL of 1% BHT were added to the previous mixture. All samples were cooled, then centrifuged at 3000 rpm for 15 min. 1 mL of the supernatant of each sample was added to 1 mL of TCA (15%) to precipitate protein, and then the samples were recentrifuged at 3000 rpm for 10 min. To each 1 ml of supernatant, 0.5 mL of TBA (0.7%) was added and boiled in a boiling water bath for 45 min. The absorbances, using a M108 Programmable Visible Range spectrophotometer (Camspec Analytical Instruments Ltd. UK), of samples were read at 532 nm against a blank reagent (Tappel and Zalkin 1959).

Determination of human seminal plasma and spermatozoa TBARS (Tappel and Zalkin 1959): Semen samples were collected from healthy subjects from a clinical laboratory after subject approval. 100 µL of tested extract were added to 100 µL of semen sample and 400 µL of HAM's reagent then centrifuged at 3000 rpm for 15 min. Sperm pallet and seminal plasma were collected separately for TBARS determination as follows: 0.5 mL of the seminal plasma was added to 1 mL TCA and centrifuged at 3000 rpm for 10 min, then the same TBARS determination method was used as described previously. For determination of sperm pallet TBARS, the pellet was mixed with 0.5 ml phosphate buffer (0.1 M, pH 7.4) and mixed with 1 mL TCA then centrifuged at 3000 rpm for 10 min, and the same TBARS determination method was used as described previously.

Experimental design and statistical analyses

All data are expressed as the mean ± standard deviation (SD). The differences were considered to be statistically significant at $P < 0.05$. Statistical analyses were performed using the unpaired Student's *t*-test and one-way analysis of variance (ANOVA) using Primer of Biostatistics (Version 5) software program.

RESULTS

The phytochemical screening of *P. capillacea*, *J. rupin* and *U. lactuca* was carried out in the methanolic extract in order to estimate their activities. *J. rupin* extract contains the highest amount of flavonoids than those of *P. capillacea* and *U. lactuca* at $P < 0.05$ (**Table 1**). On the other hand, there were no significant differences between total phenolic content in all three algae at $P < 0.05$ (**Table 1**).

The effects of our tested extracts on different enzymes activities were measured to evaluate their bioactivities. *J. rupin* and *U. lactuca* extracts enhanced the hepatic α-glucosidase activity by 30.56 and 2.78%, respectively, while *P. capillacea* extract inhibited the enzyme activity by 14.35% at $P < 0.05$. Moreover, all three extracts inhibited the activity of AChE to several degrees; as *U. lactuca* showed the highest inhibition percentage while *J. rupin* and *P. capillacea* showed the same lowest inhibition percent at $P < 0.05$ (**Table 2**). **Table 3** shows that the tested extracts had anticoagulation properties as they significantly increased PT and APTT at $P < 0.05$; *U. lactuca* had the highest anticoagulant effect while *P. capillacea* had the lowest.

All tested extracts showed antioxidant properties as they decreased the level of induced TBARS in the liver homogenate, human seminal plasma and spermatozoa. All algal extracts significantly inhibited the induction of lipid peroxidation in liver homogenate and spermatozoa by the same value at $P < 0.05$ (**Table 4**). On the other hand, *U. lactuca*

Table 1 Total phenol and flavonoid concentration of the methanolic extract of three algae.

Algae	Flavonoid concentration (mg %)	Total phenol concentration (mg%)
Ulva lactuca	8.4 ± 0.12 c	1.028 ± 0.16 a
Jania rupin	17.4 ± 0.09 a	1.234 ± 0.12 a
Pterocladia capllacea	16.75 ± 0.03 b	1.11 ± 0.08 a

Each value is the mean ± SD of triple determinations.
Within each column, values with the same letter are significantly different at $P < 0.05$.

Table 3 Effect of methanolic extract of three algae on blood coagulation tests.

Algae	% of increasing of PT	% of increasing of APPT
Ulva lactuca	102.2% ± 14.14 a	51.2% ± 16.97 b
Jania rupin	75.8% ± 00.01 b	101.2% ± 14.85 a
Pterocladia capllacea	37.4% ± 04.95 c	115.8% ± 12.02 a

Each value is the mean ± SD of triple determinations.
Within each column, values with the same letter are significantly different at $P < 0.05$.

Table 2 Effect of methanolic extract of three algae on AChE and alpha glucosidase activities.

Algae	% Inhibition of AChE	% Activation or inhibition of α-glucosidase
Ulva lactuca	83.62 ± 1.02 a	2.78 ± 1.60 (activation)
Jania rupin	79.84 ± 1.24 b	30.56 ± 7.65 (activation)
Pterocladia capllacea	74.77 ± 5.72 b	14.35 ± 10.52 (inhibition)

Each value is the mean ± SD of triple determinations.
Within each column, values with the same letter are significantly different at $P < 0.05$.

Table 4 Effect of methanolic extract of three algae on lipid peroxidation level through the percent of inhibition of TBARS in liver homogenate, seminal plasma and spermatozoa.

Algae	Liver homogenate	Seminal plasma	Spermatozoa
Ulva lactuca	90.61 ± 0.62 a	61.96 ± 6.01 a	26 ± 3.3 a
Jania rupin	90.47 ± 7.37 a	61.86 ± 6.32 a	22 ± 6.7 a
Pterocladia capllacea	82.12 ± 7.51 a	46.90 ± 2.34 b	30 ± 3.3 a

Each value is the mean ± SD of triple determinations.
Within each column, values with the same letter are significantly different at $P < 0.05$.

and *J. rupin* extracts showed the highest, and similar, antioxidant capacity at seminal plasma level (**Table 4**).

DISCUSSION

The methanolic extracts of these algae were evaluated for their effect on two important enzymes AChE enzyme (E.C.3.1.1.7) and α-glucosidase enzyme (E. C.3.2.1.20). The first enzyme is important for breaking acetylcholine (**Table 2**), which is the substrate of this enzyme that acts as a neurotransmitter in both the peripheral nervous system and the central nervous system (Perry *et al.* 1995). In abnormal activation of AChE, acetylcholine will degrade rapidly, especially in the brain, and is associated with Alzheimer's disease (AD). Some drugs that inhibit AChE are commonly used in the treatment of AD (Wilkinson *et al*. 2004). In this study we found that the extracts significantly inhibited the activity of AChE enzyme, so the algal extracts may be used for the treatment of AD. Also in agriculture AChE inhibitors are used as pesticide agents (Chttmanat *et al*. 2008).

α-Glucosidase is involved in several biological processes such as the intestinal digestion and the biosynthesis of glycoproteins. Intestinal α-glucosidases are involved in the final step of carbohydrate digestion which is absorbed from the intestine to be converted into monosaccharides (Asano 1998). α-Glucosidase is also important for producing glucose, through the glycogen catalytic pathway in the liver (Taylor *et al*. 1994), therefore its inhibitors are used as drugs for treatment of diabetes mellitus type 2 because they will decrease glucose reflux into blood (Moritoh *et al*. 2009). Our results emphasize that the *P. capillacea* extract, which inhibited α-glucosidase, can be used as a curative therapeutic compound for diabetes mellitus type 2. On the other hand, the *U. lactuca* and *J. rupin* extracts acted as α-glucosidase activators (**Table 2**). It well known that glucosidase activators are effective in hypoglycemic conditions and glycoprotein formation (Leligdowicz 2004), cellulose biosynthesis, tissue culture (Gillmor *et al*. 2002), and in the treatment of genetic disorders like Pompe's disease (Franco *et al*. 2005). So, these extracts could be used in these cases.

Furthermore, algal extracts were examined to estimate their effect on the antioxidant status in liver homogenate, seminal plasma and spermatozoa. Oxidative stress occurs as a result of increased production of destructive reactive oxygen species (ROS, including oxygen ions, free radicals and peroxides) over than the body scavenger system which consisting of enzymatic antioxidants and non-enzymatic antioxidants (Leeuwenburgh *et al*. 2001). Oxidative stress is commonly in several diseases such as steatohepatitis, diabetes, cancer and idiopathic male infertile (Chauhan *et al.* 2006). Free radicals are important in both the normal functioning and in the pathophysiology of human spermatozoa (Aitken *et al*. 1999), which rely on reduction-oxidation processes for normal functions such as hyperactivation, capacitation, and acrosome reaction (Sharma and Agarwal 1996; Aitken 1997). In idiopathic male infertile, the level of ROS are generated by sperm and seminal leukocytes within semen and produce infertility by two key mechanisms; first, they damage the sperm cell membrane, decreasing sperm motility and its ability to fuse with oocyte. Second, ROS can alter the sperm DNA, resulting in the passage of defective paternal DNA onto conceptus (Tremellen 2008). In spite of the vital role of ROS in fibrogenesis, its increase leads to hepatic damage; most hepatocellular carcinomas that take place in cirrhotic livers are due to inflammation associated with severe oxidative stress (Muriel 2009). The treatment strategies used to treat these ROS-associated diseases are based on the use of antioxidants compounds (Aitken *et al*. 1989). The most famous naturally compounds with antioxidant capacity are alkaloids, flavonoids and total phenols such as in onion and garlic, which are rich sources of alkaloids and flavonoids that have functional health benefits in the reduction of cardiovascular disease risk by lowering serum cholesterol and blood pressure (Banerjee and Maulik 2002). They have anticarcinogenic, antidiabetic, anti-platelet aggregation and anti-biotic effects (Augusti 1996; Milner 1996; Lau 1998; El-Demerdash *et al*. 2005). In this study we found that all tested algal extracts contain a high amount of flavonoids and total phenolic contents (**Table 1**) that could be providing them a high antioxidant capacity and enabling them to be used for the treatment of several diseases (Pourmorad *et al.* 2006). To prove their antioxidant capacities, algal lipid peroxidation inhibitory effects were estimated *in vitro*. Our results improved that the methanolic extract of green algae (*U. lactuca*) had the highest antioxidant activity on human seminal plasma. So we postulated that green algal extract could be used for the treatment of idiopathic male infertility and thus in reproductive technology. *Panax ginseng* (as a naturally antioxidant) has been used to treat idiopathic male infertility (Steven 2000). Furthermore, one of the greatest problems in *in vitro* fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) is the formation of ROS that could be lead to genetic mutation in sperm and finally female abortion (Agarwal 2007). So, one way to eliminate this adverse effect is to add pure antioxidant into sperm preparation medium (Twigg *et al*. 1998). Furthermore, our algal extracts showed a powerful inhibitory action against oxidative stress in liver tissue; thus, these extracts could be used in the treatment of oxidative stress-associated liver damage.

Moreover, extracts of selected algae had anticoagulant activity. Anticoagulant agents, as detected in our algal extracts, are compounds with an ability to prevent the activation of the coagulation cascade and may thus be used as a treatment for stroke and blood coagulation diseases (Savelieva 2007).

In conclusion the green algae could be used as therapeutic and industrial compounds because they have potent antioxidants, anticholinergic, antidiabetic, anticoagulant and hyperglycemic capacities.

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